



Rare coding variants in genes encoding GABA_A receptors in genetic generalised epilepsies: an exome-based case-control study

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Summary

Background Genetic generalised epilepsy is the most common type of inherited epilepsy. Despite a high concordance rate of 80% in monozygotic twins, the genetic background is still poorly understood. We aimed to investigate the burden of rare genetic variants in genetic generalised epilepsies.

Methods For this exome-based case-control study, we used three different genetic generalised epilepsy case cohorts and three independent control cohorts, all of European descent. Cases included in the study were clinically evaluated for genetic generalised epilepsy. Whole-exome sequencing was done for the discovery case cohort, a validation case cohort, and two independent control cohorts. The replication case cohort underwent targeted next-generation sequencing of the 19 known genes encoding subunits of GABA_A receptors and was compared to the respective GABA_A receptor variants of a third independent control cohort. Functional investigations were done with automated two-microelectrode voltage clamping in *Xenopus laevis* oocytes.

Findings Statistical comparison of 152 familial index cases with genetic generalised epilepsy in the discovery cohort to 549 ethnically matched controls suggested an enrichment of rare missense (Nonsyn) variants in the ensemble of 19 genes encoding GABA_A receptors in cases (odds ratio [OR] 2.40 [95% CI 1.41–4.10]; $p_{\text{Nonsyn}}=0.0014$, adjusted $p_{\text{Nonsyn}}=0.019$). Enrichment for these genes was validated in a whole-exome sequencing cohort of 357 sporadic and familial genetic generalised epilepsy cases and 1485 independent controls (OR 1.46 [95% CI 1.05–2.03]; $p_{\text{Nonsyn}}=0.0081$, adjusted $p_{\text{Nonsyn}}=0.016$). Comparison of genes encoding GABA_A receptors in the independent replication cohort of 583 familial and sporadic genetic generalised epilepsy index cases, based on candidate-gene panel sequencing, with a third independent control cohort of 635 controls confirmed the overall enrichment of rare missense variants for 15 GABA_A receptor genes in cases compared with controls (OR 1.46 [95% CI 1.02–2.08]; $p_{\text{Nonsyn}}=0.013$, adjusted $p_{\text{Nonsyn}}=0.027$). Functional studies for two selected genes (*GABRB2* and *GABRA5*) showed significant loss-of-function effects with reduced current amplitudes in four of seven tested variants compared with wild-type receptors.

Interpretation Functionally relevant variants in genes encoding GABA_A receptor subunits constitute a significant risk factor for genetic generalised epilepsy. Examination of the role of specific gene groups and pathways can disentangle the complex genetic architecture of genetic generalised epilepsy.

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Research in context

Evidence before this study

Genetic generalised epilepsies are a group of diseases with a complex inheritance, meaning that many common and rare genetic factors are probably involved in the cause of the disease, the genetic architecture of which is still largely unknown. We searched PubMed for articles published in the English language with the search terms "exome sequencing AND ion channels", "exome sequencing AND genetic generalized epilepsy", or "exome sequencing AND idiopathic generalized epilepsy" from inception until May 30, 2017. At the time we did this study, only two studies had used targeted or whole-exome sequencing in small cohorts of individuals with genetic generalised epilepsy. Neither study detected significant differences in the burden of rare genetic variants in cases versus controls. One study used a targeted Sanger sequencing approach in 237 ion channel genes in 152 cases versus 139 controls. The other study applied whole-exome sequencing to 118 individuals with juvenile myoclonic epilepsy and absence epilepsy, two of the main subphenotypes of genetic generalised epilepsy, and 242 controls. In a third study by Epi4K and the Epilepsy Phenome/Genome Project, whole exomes of 640 individuals with genetic generalised epilepsy and 3877 controls were sequenced, showing that the rate of ultra-rare deleterious variants in a group of established epilepsy genes was significantly increased; no single gene showed a mutational burden in genetic generalised epilepsy.

Added value of this study

We detected a significant difference between cases and controls in missense variants in a specific group of genes encoding all known GABA_A receptors. This finding is, to our knowledge, novel in the literature. We were able to reproduce this signal in two independent case cohorts in comparison with two independent control cohorts. We also showed the functional relevance of some of the detected variants in two GABA_A receptor subunit genes that were, until now, not known to be associated with genetic generalised epilepsy. Our study therefore provides strong evidence that variation in genes encoding GABA_A receptors confers a genetic burden in genetic generalised epilepsy. Our findings also point to the utility of burden analysis of gene sets involved in specific physiological pathways in explaining the genetics behind generalised epilepsies.

Implications of all the available evidence

Our findings, along with the study by Epi4K and the Epilepsy Phenome/Genome Project, present the first evidence of a significant genetic burden in individuals with genetic generalised epilepsy compared with controls in ultra-rare variants affecting previously identified epilepsy genes or, as suggested here, in the specific gene group of the most important inhibitory receptors in the mammalian brain.

Introduction

Gene discovery in monogenetic diseases, including familial and severe epilepsy syndromes, has revealed a steadily increasing number of disease-causing genetic defects. Unravelling the genetic architecture of complex disorders has been more difficult. Genetic generalised epilepsy comprises common epilepsies with generalised absence, myoclonic, and tonic-clonic seizures.¹ The disease has high heritability, as has been shown in twin studies.² A few single nucleotide polymorphisms in genome-wide association studies and altered copy number variations have been the major common risk factors identified so far in genetic generalised epilepsy. These risk factors, however, only explain a small part of the high heritability. Single gene defects in larger families with autosomal dominantly inherited genetic generalised epilepsy have been identified as disease-causing, such as defects in *GABRA1* or *GABRG2* encoding subunits of GABA_A receptors,^{3–5} or in *SLC2A1* encoding the glucose transporter type 1.^{6,7} Early sequencing-based candidate-gene or whole-exome sequencing studies did not reveal a significant burden of mutations in single genes or groups of genes.^{8,9} One study has shown a mutational burden of ultra-rare variants in gene sets related to epilepsy.¹⁰

We aimed to investigate the burden of rare genetic variants in familial genetic generalised epilepsy by first

testing all genes via a hypothesis-free approach, and then in hypothesis-driven disease-relevant gene sets. Significant findings were validated and replicated in additional cohorts and functional studies done for selected variants.

Methods

Study design and participants

For this exome-based case-control study, we used three different genetic generalised epilepsy case cohorts and three independent control cohorts, all of European descent. The genetic generalised epilepsy diagnoses included mainly the classical four phenotypes of childhood or juvenile absence epilepsy, juvenile myoclonic epilepsy, or genetic generalised epilepsy with generalised tonic-clonic seizures alone; we included a few cases of early-onset absence epilepsy (defined as beginning in children <3 years), epilepsy with myoclonic absences, and up to 30% of cases with unclassified genetic generalised epilepsy, since these entities are, in our view, close to classical genetic generalised epilepsy. Results of a large study have suggested that early-onset absence epilepsy is likely to be genetically similar to classical childhood absence epilepsy,¹¹ that epilepsy with myoclonic absences might also have genetic overlaps with genetic generalised epilepsy,¹² and that in family studies both well classified and unclassified genetic generalised epilepsy cases are

often found in the same pedigrees (see appendix 1 for detailed phenotypes in all cohorts). The first case cohort, the discovery whole-exome sequencing case cohort, comprised 152 individuals (after quality control) with genetic generalised epilepsy from multiplex families, collected by the Epicure and the EuroEPINOMICS CoGIE consortia. 143 (94%) cases were derived from multiplex families with at least two affected family members, of which 76 families had three or more affected members (appendix 1). The second case cohort, the validation cohort, consisted of 357 genetic generalised epilepsy cases (after quality control) collected by the EpiPGX consortium. 92 (26%) cases were derived from families with at least two affected members. 131 (37%) cases were sporadic and for the remaining 134 (37%) cases the familial history was not known (appendix 1). Two independent whole-exome sequencing control cohorts were obtained from two separately sequenced cohorts (termed A and B) from the Rotterdam study,^{13,14} which were matched for ethnicity (appendix 1). These were population-based controls recruited at an age of 55 years or older. Controls were chosen so that the distribution of sexes was similar to that of corresponding cases. The third case cohort, the replication cohort, was collected in Quebec, Canada, and in Europe for GABA_A receptor gene panel sequencing and consisted of 583 cases (after quality control). 142 (24%) were derived from families with at least two affected individuals and 47 (8%) additional individuals reported a positive family history. A third independent cohort of population-based controls (cohort C) was obtained from the UK10K project¹⁵ (appendix 1). UK10K control samples were of self-reported European ethnicity and were processed to remove outlier samples (appendix 1). 635 (after final quality control) ethnically matched individuals were selected from the control cohort C.

Genetic generalised epilepsies were diagnosed on clinical grounds (ie, on the basis of clinical interview, neurological examination, EEG recordings, and available imaging data), by experienced epileptologists, according to International League Against Epilepsy (ILAE) classifications at the time of diagnosis and recruitment. All patients were diagnosed on the basis of the older criteria but still met the criteria according to the latest version from 2017.¹ Written informed consent was obtained from all participants or their relatives. The study was approved by local ethical committees. One affected individual of each family was selected for sequencing.

Procedures

For the discovery stage, paired-end whole-exome sequencing of cases and controls (cohorts A and B) was done with the Illumina HiSeq 2000 by use of the EZ Human Exome Library v2.0 kit (NimbleGen, Madison, WI, USA). Cases and controls were sequenced at different locations: cases at the Cologne Center for Genomics (Köln, Germany) and the control cohort A in

Rotterdam (Netherlands).¹³ Sequencing adapters were trimmed and samples with less than 30× mean depth or less than 70% total exome coverage at 20× mean depth of coverage were excluded from further analysis. For the validation cohort, whole-exome sequencing was done at deCODE genetics (Reykjavik, Iceland) on the Illumina HiSeq 2500 with the Nextera Rapid Capture Expanded Exome kit (Illumina, San Diego, CA, USA). The Rotterdam control cohort B¹⁴ was sequenced in the same way as cohort A. Variant calling was done by use of the Genome Analysis Toolkit (GATK)¹⁶ best practices pipeline with the GRCh37 human reference genome (appendix 1). To exclude low-quality variants, we did additional filtering based on quality metrics of individual genotypes, using read depth and genotype quality as the filtering criteria. We excluded any variant position with a mean depth of less than 10 in either cases or controls. For all whole-exome sequencing samples, the same exome regions file from the EZ Human Exome Library v2.0 kit was used. Only samples with more than 30× mean coverage or more than 70% of the exome intervals covered by at least 20× mean coverage were included for analysis (appendix 1).

For the replication cohort, 19 genes encoding known subunits of GABA_A receptors were selected for deep sequencing (appendix 1). After quality trimming, sequencing reads were mapped against the GRCh37 human reference genome with the GATK¹⁶ suite and the MUGQIC pipelines. Data from control cohort C were processed with the same pipelines as cases. Coverage comparisons were made to keep bases covered in at least 95% of individuals as well as in the control cohort.

RefSeq gene annotation information was used for classification into missense (Nonsyn) and synonymous (Syn) variants and to filter for rare (minor allele frequency <0.5%) variants by use of the ExAC database¹⁷ (appendix 1).

Population stratification

For both whole-exome sequencing datasets, selected common variants from each study were chosen and principal-components analysis was applied to assess potential population substructure separately for each case-control cohort, using implementation in Eigenstrat.¹⁸ Population outliers were defined as an SD of more than 3 based on the first ten principal components and excluded from further analysis (appendix 1).

Statistical analysis

Because of the limited sample size, hypothesis-free single gene collapsing analysis for the discovery stage was done with the combined and multivariate collapsing¹⁹ (CMC) method with a two-sided Fisher's exact test, as implemented in the Exact CMC method in rvtests²⁰ (appendix 2). p values for single-gene collapsing tests were corrected for multiple testing by use of the Bonferroni method for 18 668 protein-coding genes.

See Online for appendix 1

For more on MUGQIC pipelines see https://bitbucket.org/mugqic/mugqic_pipelines

For more on the UK10K project see <https://www.uk10k.org/>

See Online for appendix 2

For all three stages, hypothesis-driven gene set collapsing tests were done with the regression-based two-sided SKAT-O method,²¹ as implemented in *rvtests*.²⁰ For the two whole-exome sequencing cohorts, we included sex and the first ten principal components from the Eigenstrat analysis as covariates to account for possible sex and population substructure effects. Gene-set collapsing tests were applied separately to missense and to synonymous variants. Three disease-related and four process-specific gene sets related to genetic generalised epilepsy were constructed (appendix 1). To control the family-wise error rate, we applied Holm's correction for multiple testing of 14 hypotheses (seven gene sets for missense and synonymous variants each) in the discovery cohort, while the correction was done for only two hypotheses in both the validation and the replication cohort, since only the GABA_A receptor gene set was carried forward (appendix 1). The odds ratio (OR) for a given gene set was ascertained by comparing the presence of qualifying rare (missense or synonymous) variants in all genes within each gene set between cases and controls by use of the R package 'fmsb'.

Functional experiments were done with automated two-microelectrode voltage clamping in *Xenopus laevis* oocytes. All methods for functional studies have been described previously^{21,22} (appendix 1).

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The authors had full access to the data in the study and had final responsibility for the decision to submit for publication.

Results

We first did whole-exome sequencing in a discovery cohort of 238 independent, mainly familial cases of classical forms of genetic generalised epilepsy (childhood or juvenile absence epilepsy, juvenile myoclonic epilepsy, or genetic generalised epilepsy with generalised tonic-clonic seizures alone). As controls, we used the ethnically matched population control cohort A from the Rotterdam study (appendix 1),¹³ which used the same enrichment and sequencing procedures, albeit with a somewhat lower coverage than that of the discovery cohort. After quality control and population outlier removal, the final dataset consisted of 152 unrelated genetic generalised epilepsy and 549 control samples. From 701 samples in the final dataset, 204023 exonic and splice site variants were called. The mean exonic transition/transversion ratio was 3.46, indicating good data quality. 93893 missense and 55170 synonymous variants constituted the analysis dataset (appendix 1). When testing all RefSeq genes separately for association via a hypothesis-free approach, we did not identify genes enriched for any variant type (appendix 1). Therefore, we

applied an independent hypothesis-driven analysis by testing the enrichment of rare variants in seven gene sets related to epilepsy or underlying molecular processes, representing all voltage-gated cation channels, all excitatory postsynaptic receptors, all GABA_A receptors as the main inhibitory postsynaptic receptors, the GABAergic pathway more broadly (since such genes have been associated specifically with generalised epilepsies), and genes associated with generalised epilepsies, epileptic encephalopathies, or focal epilepsies (appendix 1). We tested separately for each variant type; synonymous variants were expected to show no difference between cases and controls. In this hypothesis-driven analysis, we found an enrichment for missense variants in the GABA_A receptor gene set, which was significant when correcting for multiple comparisons in the seven gene sets for both missense and synonymous variants (ie, 14 altogether) by use of the two-sided SKAT-O test (19 genes, $p_{\text{Nonsyn}}=0.0014$, adjusted $p_{\text{Nonsyn}}=0.019$, OR 2.40 [95% CI 1.41–4.10]; table). However, the GABA_A receptor gene set did not reach study-wide significance after correcting for all single genes tested before. None of the other gene sets showed a significantly increased burden of rare variants. Synonymous variants, used as a negative control, did not show significant enrichment in any of the gene sets (table; appendix 2).

The validation cohort consisted of 724 individuals with genetic generalised epilepsy from six European countries. 268 (37%) cases were sporadic, while 265 (37%) were of unknown familial history, and all were diagnosed with classical forms of genetic generalised epilepsy (appendix 1). For comparison, control cohort B from the Rotterdam study¹⁴ was used. After applying the same quality control steps as for the discovery cohort, the dataset consisted of 357 unrelated genetic generalised epilepsy and 1485 control samples. Consistent with the discovery stage, in the validation cohort we observed significant enrichment of rare missense variants in the GABA_A receptor gene set in cases after multiple-testing correction for two sets of variants (Nonsyn, Syn; $p_{\text{Nonsyn}}=0.0081$, adjusted $p_{\text{Nonsyn}}=0.016$, OR 1.46 [95% CI 1.05–2.03]) with the SKAT-O test (table; appendix 1). Synonymous variants showed no significant enrichment.

For the independent replication cohort, consisting of 631 cases with familial or sporadic genetic generalised epilepsy (appendix 1), we designed a targeted enrichment panel comprising all 19 genes encoding GABA_A receptors. *GABRR3* was excluded for quality control reasons. Since no genotype data for the cases were available and therefore sex-based quality control could not be done, the burden analysis was restricted to the remaining 15 autosomal GABA_A receptor genes. We obtained control samples from the UK10K project and selected 639 individuals after sample quality control. Additional variant quality control led to a final dataset of 583 unrelated cases and 635 controls. We replicated significant enrichment of rare missense variants for 15 GABA_A receptor genes in cases

	Variant class	Number of variants	Number of cases	Number of controls	OR (95% CI)	SKAT-O p value unadjusted	SKAT-O p value adjusted
EuroEPINOMICS CoGIE (discovery cohort)							
Epileptic encephalopathies	Syn	284	152	549	1.25 (0.87–1.80)	0.292299	1
Excitatory receptors	Syn	151	152	549	1.43 (0.97–2.11)	0.212472	1
Focal epilepsies	Syn	110	152	549	1.07 (0.68–1.66)	0.416506	1
GABAergic pathway	Syn	444	152	549	1.33 (0.92–1.92)	0.0628653	0.8172489
GABA _A receptors	Syn	64	152	549	1.37 (0.78–2.40)	0.574707	1
Generalised epilepsies	Syn	177	152	549	0.99 (0.67–1.47)	0.237372	1
Voltage-gated ion channels	Syn	471	152	549	1.19 (0.81–1.74)	0.771146	1
Epileptic encephalopathies	Nonsyn	259	152	549	1.42 (0.98–2.07)	0.528544	1
Excitatory receptors	Nonsyn	241	152	549	1.01 (0.69–1.48)	0.708729	1
Focal epilepsies	Nonsyn	142	152	549	1.42 (0.93–2.17)	0.523553	1
GABAergic pathway	Nonsyn	564	152	549	1.66 (1.12–2.46)	0.442513	1
GABA _A receptors*	Nonsyn	63	152	549	2.40 (1.41–4.10)	0.0013633	0.0190862
Generalised epilepsies	Nonsyn	194	152	549	2.17 (1.49–3.17)	0.166314	1
Voltage-gated ion channels	Nonsyn	664	152	549	1.78 (1.17–2.70)	0.601852	1
EpiPGX (validation cohort)							
GABA _A receptors	Syn	99	357	1485	0.82 (0.54–1.25)	0.587127	0.58712700
GABA _A receptors*	Nonsyn	107	357	1485	1.46 (1.05–2.03)	0.00805992	0.01611984
GABA panel (replication cohort)							
GABA _A receptors†	Syn	86	583	635	0.87 (0.60–1.25)	0.0613778	0.0613778
GABA _A receptors*†	Nonsyn	85	583	635	1.46 (1.02–2.08)	0.0133277	0.0266554

For all cohorts, the corrected and uncorrected SKAT-O p values for different gene sets and variant classes are given, together with the number of qualifying variants per gene set and the number of cases and controls used for testing. Syn=synonymous variants. Nonsyn=missense variants. OR=odds ratio. OR was calculated for only qualifying rare variants (missense or synonymous) over all genes per gene set. Gene sets are described in appendix 1. *Enriched gene sets after applying a false discovery rate threshold of 0.05. †In the replication cohort, we used a reduced set of 15 autosomal GABA_A receptor genes because of sex-based quality control reasons.

Table: Gene-set burden analysis for rare coding variants in three genetic generalised epilepsy cohorts

compared with controls ($p_{\text{Nonsyn}}=0.013$, adjusted $p_{\text{Nonsyn}}=0.027$, OR 1.46 [95% CI 1.02–2.08]; table) by use of the SKAT-O test. Synonymous variants were not significantly enriched.

All detected case-only variants are provided in appendix 1. Case-only rare missense variants were found across all GABA_A receptor genes except for *GABRR3* (appendix 1).

The combination of two α_1 subunits, two β_2 subunits, and one γ_2 subunit (encoded by *GABRA1*, *GABRB2*, and *GABRG2*) represents the most common form of a functional GABA_A receptor in the brain,²³ and variants in *GABRA1* and *GABRG2* have been shown to have an important role in familial genetic generalised epilepsy, febrile seizures, and epileptic encephalopathies.^{4,5,22,24–27} The observed enrichment of missense variants was not driven by variants in the two epilepsy genes, *GABRA1* and *GABRG2*; the signal was no longer significant after reducing the analysis to those two genes (appendix 1). Instead, the qualifying variants were evenly distributed across all genes encoding GABA_A receptors.

The α_5 subunit (encoded by *GABRA5*) is thought to mediate extrasynaptic tonic inhibition,²⁸ and tonic inhibition has been described to be altered in genetic mouse models of epilepsy.^{29,30} *GABRB2* and *GABRA5* have not previously been associated with genetic

generalised epilepsy, although *GABRB2* mutations have been described in patients with intellectual disability and epilepsy.^{31–33} For functional studies, we selected seven missense variants in *GABRB2* and *GABRA5* (two genes not previously associated with genetic generalised epilepsy; appendix 1) identified in families with genetic generalised epilepsy for electrophysiological studies in *X laevis* oocytes. All selected variants were confirmed by Sanger sequencing. Five of these variants were selected since they co-segregated with the phenotype in nuclear families. Another variant (p.Arg3Ser) was found in three different French-Canadian pedigrees, so we hypothesised that this could be a more common causal variant in a specific population (figures 1A, 2A). The last variant, p.Pro453Leu, did not co-segregate, but was selected as an additional *GABRA5* variant localised in a different protein region (the C-terminus) from the other variants. All missense variants were predicted to be deleterious by at least three of seven prediction tools and were highly conserved (appendix 1). Three of these variants were consistently of ultra-low frequency in the European population in different public databases (1000G, ExAC, and gnomAD; appendix 1). Localisation of the variants is shown in figures 1B and 2B.

After application of 1 mM GABA, we observed a significant reduction in current amplitudes of GABA_A

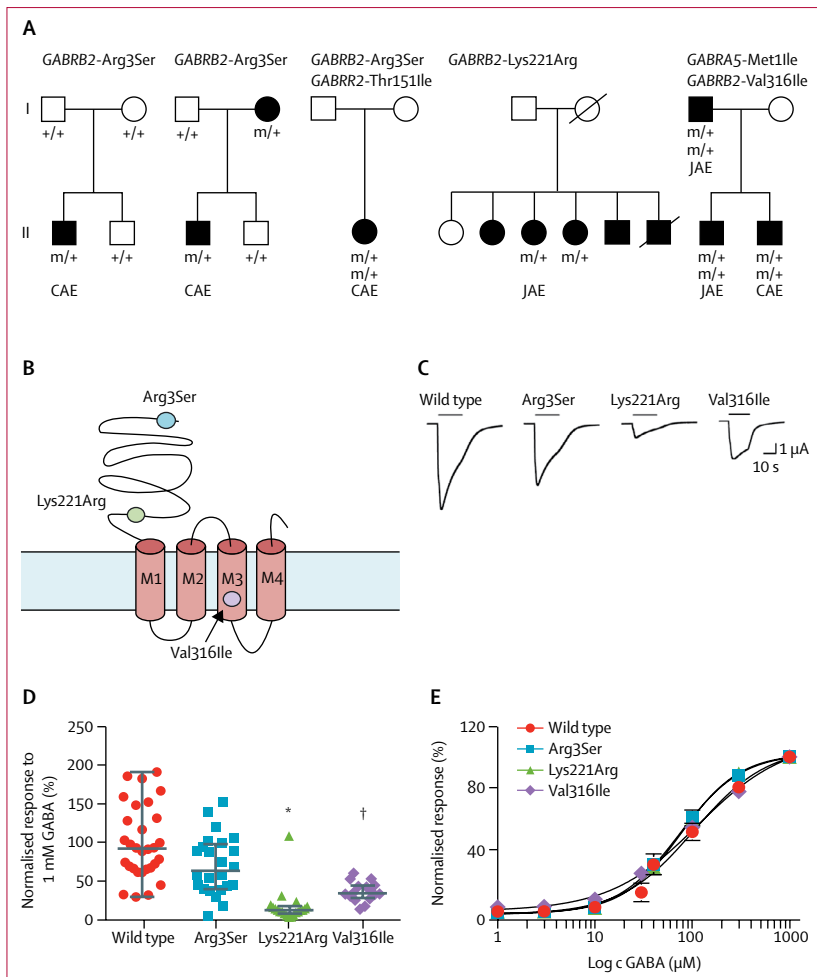


Figure 1: Characterisation of GABRB2 missense variants associated with genetic generalised epilepsy
 GABRB2 mutations associated with genetic generalised epilepsy. (A) Family pedigrees. Squares represent male individuals, circles represent female individuals, filled symbols denote affected individuals, +/+ denotes two wild-type alleles, and m/+ denotes heterozygous mutant and wild-type alleles. Diagonal lines indicate deceased individuals. (B) Schematic representation of the β_2 subunit of GABA_A receptors and predicted positions of the Arg3Ser and Lys221Arg mutations located in the N-terminal domain and Val316Ile located in the third transmembrane domain (M3). (C) Examples of GABA-induced currents after 1 mM GABA application for wild-type, Arg3Ser, Lys221Arg, and Val316Ile mutations. (D) Current responses normalised to 1 mM GABA application for wild-type (n=30), Arg3Ser (n=24), Lys221Arg (n=21), and Val316Ile (n=16). (E) Dose-response curve for $\alpha 1\beta 2\gamma 2s$ wild-type (n=30), Arg3Ser (n=14), Lys221Arg (n=10), and Val316Ile (n=7) obtained via application of different GABA concentrations and normalisation to the maximal GABA response for each cell. CAE=childhood absence epilepsy. JAE=juvenile absence epilepsy. *p<0.0001, †p=0.0002, Kruskal Wallis test, with Dunn's comparison test.

receptors containing either p.Lys221Arg or p.Val316Ile variants in the β_2 subunit, and p.Met1Ile or p.Ser238Asn in the α_5 subunit, in comparison with respective compositions of wild-type receptors. No significant reductions were observed for p.Arg3Ser in the β_2 subunit and for p.Glu243Lys or p.Pro453Leu in the α_5 subunit (figures 1C, 1D, 2C, 2D). GABA sensitivity was similar between wild-type receptors and all of the variant-carrying receptors (figures 1E, 2E). Thus, four of seven variants led to a loss of receptor function predicting postsynaptic or extrasynaptic neuronal disinhibition.

Discussion

We found replicable enrichment of rare missense variants in genes encoding GABA_A receptor subunits in three independent cohorts, which together comprised more than 1000 genetic generalised epilepsy index cases (both familial and sporadic). Four selected variants in two genes (GABRB2 and GABRA5) previously not associated with genetic generalised epilepsy changed receptor function and co-segregated in nuclear families, suggesting an important contribution to the genetic generalised epilepsy phenotype. Previous studies in smaller cohorts, either investigating all genes encoding ion channels⁸ or with single-gene collapsing tests based on whole exomes, did not show a significant excess of variants in cases versus controls.⁹ The differences between previous studies and our study could be explained by the larger sample size in our study across all cohorts and by the fact that we tested different gene sets that had not been considered before. In another study,¹⁰ a similar effect was shown for ultra-rare deleterious variants in gene sets comprising known epilepsy genes or genes associated with epileptic encephalopathies (GABA_A receptor genes were not investigated as a separate gene set). Because of our smaller sample size and the associated low number of ultra-rare variants, we chose a different approach, considering all variants with a minor allele frequency less than 0.5%, which revealed significant genetic signals in studies of other diseases.^{34–36} Both the Epi4k study¹⁰ and our study did not identify single genes with a genome-wide significant burden of rare variants in individuals with genetic generalised epilepsy. Future studies should consider combining different cohorts to increase statistical power for such analyses, which might shed more light on the complex genetic architecture of genetic generalised epilepsy.

One limitation of our study is that the cohorts, because of funding restrictions of the individual projects, were sequenced at different locations by use of different technologies. Combining and analysing such data in an unbiased way is still a major challenge. To minimise any bias, we used only those regions after quality control that were available for all samples. An a-priori selection bias for the targeted genes yielding false significance can also not be completely ruled out. Careful selection of gene sets was based on biological and published evidence and did not change the selection afterwards, which should minimise selection bias and associated false-positive findings. Furthermore, we applied stringent quality control and consistent processing of all datasets, and altogether had three independent case and control datasets for discovery, validation, and replication of results. Neutral signals emerging from synonymous variants across all case-control datasets suggest that we controlled for any major population structure or other confounding factor.

All four variants inducing significantly reduced current amplitudes co-segregated with the disease phenotype in respective pedigrees (figures 1A, 2A), corroborating their

pathophysiological contribution. In two families, we observed co-segregating variants in two different GABA_A receptor subunits: p.Val316Ile in the β_2 subunit and p.Met1Ile in the α_5 subunit co-occurred in the same nuclear family, and p.Glu243Lys in the α_5 subunit was accompanied by a deleterious frameshift mutation in *GABRG2* in another family (figures 1A, 2A). We did not see a significant change in GABA-evoked currents for p.Glu243Lys, suggesting that *GABRG2* might be a dominant disease-contributing gene in this family. However, as genetic generalised epilepsy only occurs in individuals carrying variants in both genes, we cannot exclude that p.E243K contributes to the phenotype with a more subtle functional change that could not be detected in our assay. Variants with altered receptor function were all located in the N-terminus containing GABA-binding sites or in the pore region. p.Met1Ile suppresses the start codon such that translation starts six aminoacids later, which shortens the signalling peptide consisting of the first 20 aminoacids. Although the peptide is removed and not part of the mature GABA_A receptor in the plasma membrane, this alteration could still affect protein biogenesis, leading to reduced expression of functional receptors. p.Arg3Ser, which also affects the signalling peptide, and p.Pro453Leu, located in the functionally less relevant C-terminus, did not lead to a significant change in receptor function. p.Arg3Ser recurred in three French-Canadian families and p.Pro453Leu was detected in only one of several affected members of a larger family, indicating that these variants might represent benign polymorphisms.

One of the variants we functionally examined in our study (p.Val316Ile in *GABRB2*) has since been identified as a de-novo mutation in a cohort of severe developmental and epileptic encephalopathies by use of whole-genome sequencing of parent-patient trios.³² This finding corroborates the pathogenicity of this variant. Association of genetic variants with different phenotypes is well known as pleiotropy and has also been described in other genes encoding GABA_A receptors,^{3,23} including large phenotypic variability within one extended pedigree.³

We have also characterised the variant p.Thr336Met in *GABRA3*, which was detected in our discovery cohort (appendix 1), as part of another study in which we identified *GABRA3* as a new epilepsy gene associated with highly heterogeneous epileptic phenotypes, including asymptomatic variant carriers.³⁷ This variant also causes a severe loss-of-function effect but does not co-segregate in the respective pedigree, so other factors must contribute to the genetic generalised epilepsy at least in two family members. Although co-segregation is a strong indicator for the pathogenicity of genetic variants, genetic generalised epilepsy is a common disease with a complex inheritance. Variants in genes encoding GABA_A receptors could therefore still contribute to the disease, whereas other family members not carrying the respective variants must have other causes of epilepsy. Similarly, copy number variations

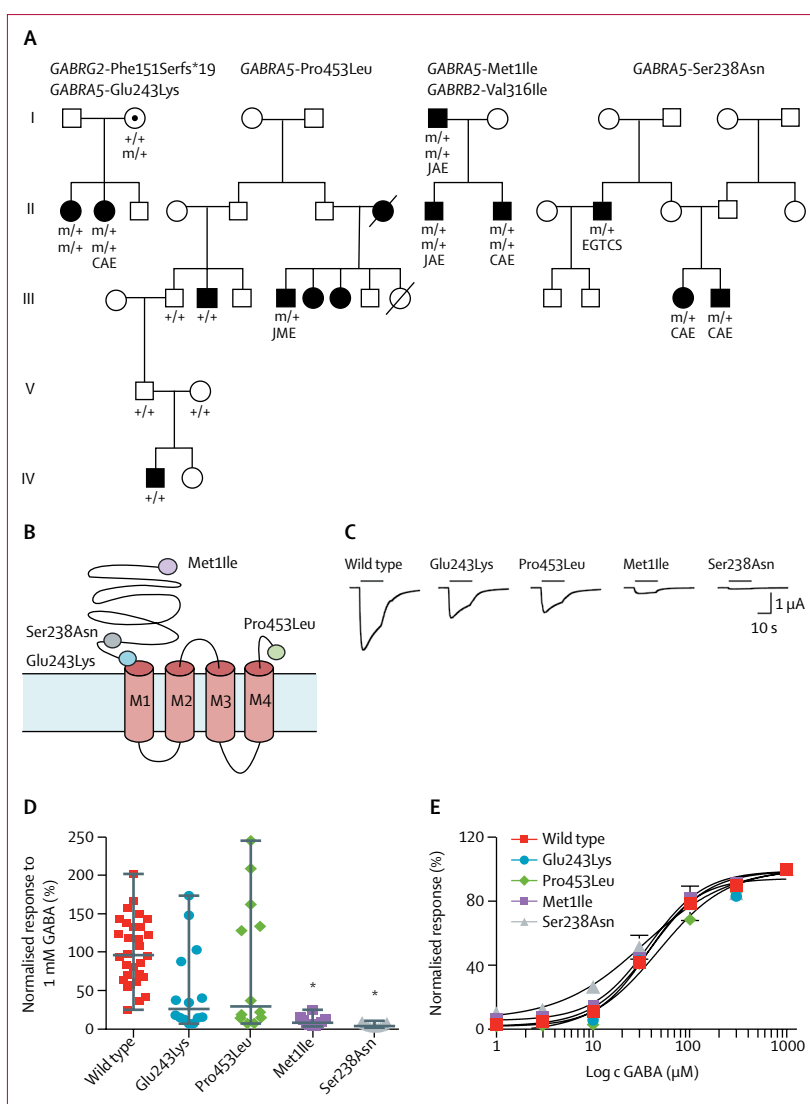


Figure 2: Characterisation of GABRA5 missense variants associated with genetic generalised epilepsy
GABRA5 mutations associated with genetic generalised epilepsy. (A) Family pedigrees. Squares represent male individuals, circles represent female individuals, filled symbols denote affected individuals, +/+ denotes two wild-type alleles, and m/+ denotes heterozygous mutant and wild-type alleles. Diagonal lines indicate deceased individuals. (B) Schematic representation of the α_5 subunit of GABA_A receptors and predicted positions of the Met1Ile, Ser238Asn, and Glu243Lys mutations located in the N-terminal domain and Pro453Leu located in the C-terminal domain. (C) Examples of GABA-induced currents after application of 1 mM GABA for wild-type, Met1Ile, Ser238Asn, Glu243Lys, and Pro453Leu mutations. (D) Normalised current responses to 1 mM GABA application for wild-type (n=43), Met1Ile (n=10), Ser238Asn (n=13), Glu243Lys (n=14), and Pro453Leu (n=11). (E) Dose-response curve for $\alpha\beta\gamma 2s$ wild-type (n=37), Met1Ile (n=15), Ser238Asn (n=11), Glu243Lys (n=8), and Pro453Leu (n=8) obtained after application of different GABA concentrations and normalisation to the maximal GABA response for each cell. CAE=childhood absence epilepsy. JME=juvenile myoclonic epilepsy. JAE=juvenile absence epilepsy. EGTCS=genetic generalised epilepsy with generalised tonic clonic seizures alone. *p<0.0001, Kruskal Wallis test, with Dunn's comparison test.

often do not co-segregate within nuclear families but have been replicated as a significant risk factor for genetic generalised epilepsy.³⁸⁻⁴¹ Given the reproducibility of our results, as well as co-segregation and functional evidence, many but not all of the detected variants might contribute to genetic generalised epilepsy in our three

cohorts. This disease-relevant contribution could range from a major gene effect—as observed in so-called monogenic mendelian epilepsies—to relatively small effect sizes in the variant carriers, depending on the amount of the electrophysiological dysfunction and other unknown factors, such as the genetic background. Overall, we consider the enrichment of GABA_A receptor variants in cases versus controls to be a significant risk factor for development of genetic generalised epilepsy.

Lastly, our results indicate a genetic overlap among rare and common forms of epilepsy, since there is increasing evidence that de-novo variants in genes encoding GABA_A receptors cause severe forms of epileptic encephalopathies^{22–26,37,42} and these findings re-iterate a central role of GABAergic mechanisms in generalised epilepsies.^{3–5,23–25,27–31,43,44}

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HL, RolK, PC, FZ, SM, PN, PM, and SG designed the study. FB, SW, PL-T, CM MG, SB, PS, HC, AS, KE, RB, RolK, RSM, HH, HM, IH, WSK, YGW, SW, PdJ, SMS, SS, RN, SF, AC, MSV, DKN, BB, UO, NB, KMK, FR, DKN, FD, LC, AL, RD, JFC, CCW, GA, TS, ELG, BPCK, FZ, PC, HL, GJS, PA, BF, MRJ, AGM, BBe, JWS, AA, MMc, GLC, ND, CD, MK, FZi, and the Epicure, EuroEPINOMICS CoGIE, and the EpiPGX consortia recruited and phenotyped participants. JvR, RK, AI, and AGU recruited, phenotyped, and analysed the individuals in the Rotterdam cohort. PM, SG, DRB, SaP, JS, CDK, MT, AP, MI, RGL, SB, CM, HT, JA, KJ, AKR, WJ, DL, ELG, JMS, BPCK, AP, AEL, MN, PN, FZ, PC, RolK, and HL did or supervised genetic studies (Sanger or whole-exome sequencing, or data analysis). MH, CEN, JK, RR, SC, BT, IDC, CAR, SS, SP, MM, SM, and HL did or supervised functional studies. PM, SG, MH, DRB, JS, SW, and HL wrote the manuscript. All authors revised the manuscript.

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Declaration of interests

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